bcl-2 Modulation of Apoptosis Induced by Anticancer Drugs: Resistance to Thymidylate Stress Is Independent of Classical Resistance Pathways


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ABSTRACT

The hypothesis was tested that expression of bcl-2 could provide protection against apoptosis induced by cytotoxic drugs via a mechanism which was different from the classical determinants of drug resistance. Sensitivity and resistance to inhibitors of thymidylate synthase (EC 2.1.1.45) were chosen for study since these drugs have a well-defined and quantifiable locus of action with similarly well defined biochemical sequelae resulting from enzyme inhibition. Human lymphoma cells transfected with the vector alone readily underwent apoptosis after a 36-h exposure to various drugs. For example, 5-fluorodeoxyuridine (0.1 μM) induced 67% apoptosis in vector control cells 24 h after removal of the drug. In contrast, cells treated under identical conditions, which expressed the bcl-2 protein, showed only basal levels of apoptosis (8%), with no significant fall in viability. Similar results were obtained using two quinazoline-based inhibitors of thymidylate synthase, N10-propargyl-5,8-dидеазазофицид (CB3717) and ICI M247496. Determinants of resistance to these three drugs were investigated. Analysis of the cell cycle, thymidylate synthase levels, and activity showed these to be unchanged by expression of bcl-2. Addition of the drugs brought about equivalent inhibition of proliferation in the presence or absence of bcl-2 expression. 5-Fluorodeoxyuridine treatment reduced TTP synthesis, induced strand breaks in nascent DNA, measured by alkaline elution, and increased the synthesis of thymidylate synthase; these changes preceded the onset of apoptosis and were identical in the vector controls and bcl-2 transfectants. Resistance to thymidylate stress in bcl-2-expressing cells therefore occurred by a mechanism different from those which classically define resistance to this type of cytotoxic drug.

INTRODUCTION

The major cancers of humans remain largely resistant to the effects of chemotherapy (1, 2). Survival rates for many cancers have therefore not improved markedly over the past decades (3). Although understanding of some of the underlying mechanisms for this resistance has been gained (4, 5), the question of why some types of cancer cells are inherently resistant to therapy, and are pleiotropically drug resistant, has not been answered. Pleiotropic drug resistance reflects the differentiated phenotype of the tumor and/or its cell of origin, where certain types of tumor are inherently sensitive and others resistant.

It is possible that some determinants of inherent drug sensitivity and resistance may be independent of those which involve the formation of the drug-target complex and its characteristic and immediate biochemical sequelae. This idea is supported by the observation that a wide variety of antitumor drugs reportedly induce a conserved mode of active cell death, termed apoptosis, in susceptible cells (reviewed in Refs. 6 and 7). This type of cell death can be modulated by the expression of certain genes so that, independently of the type of drug initiating it, it may be attenuated. The gene bcl-2 inhibits apoptosis stimulated by a variety of stressful stimuli which induce apoptosis, including some antitumor drugs (8–14). Expression of bcl-2 may therefore make cells resistant to drug-induced apoptosis by a completely novel mechanism affecting events "downstream" of the initiation of toxicity brought about by a drug. This has been implied, but not proven, in recently published studies which have shown bcl-2 expression to provide resistance to a number of cytotoxic drugs and radiation (11–14).

Inhibition of drug-induced apoptosis by bcl-2 could arise from some modulation of classical mechanisms of drug resistance, such as a change in the expression of drug targets, changes in cellular pharmacodynamics such as in drug transport across the plasma membrane, or changes in the capacity of a cell to repair damage induced by the drug. The resistance provided by bcl-2 expression extends across a spectrum of agents, which supports the idea that conserved downstream events leading to cell death are being modulated. Nevertheless, the possibility that the survival advantage provided by bcl-2 expression to drug-treated cells might involve elements of a classical type of drug resistance mechanism has not been excluded, simply because mechanisms have not been investigated. For example, in a study of the survival advantage provided to murine S49.1 lymphoma cells (12) treated with dexamethasone, the expression of glucocorticoid receptor was not altered by transfection and expression of bcl-2. However, it was not possible to establish whether bcl-2 modulated the lethal events that are initiated after interaction of this receptor with dexamethasone, since these events are not established. Additionally, there are cells with fully functional glucocorticoid receptors but which are nevertheless resistant to dexamethasone (15). Similarly, the observation of bcl-2-induced resistance to a single, low concentration of the topoisomerase II inhibitor etoposide could have resulted from modulation of either some property of topoisomerase II or a DNA repair process, neither of which was investigated (14).

Many drugs are promiscuous in their interactions with the cell, for example, the reactive alkylating agents, and it is difficult to be certain as to their precise locus of action and therefore what parameters of sensitivity bcl-2 expression may change. In order to investigate in detail whether bcl-2 affects classical determinants of drug resistance, we chose to study inhibitors of the enzyme TS3 because the enzyme is readily quantifiable in amount (16), its activity is readily measured (17), and there are a variety of potent and highly specific inhibitors of the enzyme, including novel quinazolines (reviewed in Refs. 18 and 19). Additionally, the various inhibitors of TS have pharmaceutical profiles which differ with respect to their transport across the cell membrane, their intracellular metabolism, and their intracellular fate. This allows investigation of any possible changes in the cellular pharmacodynamics of these agents which might be brought about by the expression of bcl-2. The TS enzyme catalyzes the methylation of dUMP to TMP so that the consequences of the inhibition of the enzyme are the reduction of pools of TTP (20). The result of this thymidylate stress is the introduction of strand breaks into nascent DNA, apparently because of the misincorporation of dUTP (21, 22). The major cancers of humans remain largely resistant to the effects of chemotherapy (1, 2). Survival rates for many cancers have therefore not improved markedly over the past decades (3). Although understanding of some of the underlying mechanisms for this resistance has been gained (4, 5), the question of why some types of cancer cells are inherently resistant to therapy, and are pleiotropically drug resistant, has not been answered. Pleiotropic drug resistance reflects the differentiated phenotype of the tumor and/or its cell of origin, where certain types of tumor are inherently sensitive and others resistant.

It is possible that some determinants of inherent drug sensitivity and resistance may be independent of those which involve the formation of the drug-target complex and its characteristic and immediate biochemical sequelae. This idea is supported by the observation that a wide variety of antitumor drugs reportedly induce a conserved mode of active cell death, termed apoptosis, in susceptible cells (reviewed in Refs. 6 and 7). This type of cell death can be modulated by the expression of certain genes so that, independently of the type of drug initiating it, it may be attenuated. The gene bcl-2 inhibits apoptosis stimulated by a variety of stressful stimuli which induce apoptosis, including some antitumor drugs (8–14). Expression of bcl-2 may therefore make cells resistant to drug-induced apoptosis by a completely novel mechanism affecting events "downstream" of the initiation of toxicity brought about by a drug. This has been implied, but not proven, in recently published studies which have shown bcl-2 expression to provide resistance to a number of cytotoxic drugs and radiation (11–14).

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Using a human B-lymphoma cell line we have found that transfection and expression of the bcl-2 gene provide the cells with a distinct survival advantage after imposition of a thymidylate stress. There were, however, no changes in any of the parameters described above, which are considered to determine sensitivity or resistance to this class of cytotoxic drug. This endorses proposals for the presence of novel mechanisms of drug resistance which are downstream of the events associated with drug target interactions and their close sequelae.

MATERIALS AND METHODS

Materials. All reagents, unless otherwise specified, were from Sigma Ltd., or from BDH UK (Poole). Radiochemicals were from Amersham International (United Kingdom).

Cell Culture. The cell lines used were derived from an EBV genome-positive Burkitt's lymphoma, MUTU-BL. Cloned sublines were selected which stably retained the phenotype of the original biopsy cells (Group I MUTU-BL), including the ability to undergo apoptosis (24–26) and lack of detectable bcl-2 protein expression (25). bcl-2 transfects of MUTU-BL cells were derived as described elsewhere (27). Briefly, cells were transfected by electroporation in the presence of the pcΔ-bcl-2 plasmid constructed by Tsuchimoto (28). Control transfectants containing vector alone (pcΔ-SV2) were derived in parallel. Transfectants were selected using G418 (Geneticin, Sigma), and bcl-2 expression was monitored by immunoblotting and immunofluorescence/flow cytometry using the bcl-2-specific monoclonal antibody BCL-2/124 kindly provided by Prof. D. Y. Mason (Oxford, United Kingdom). Clones selected for this study were the control clone 179-SV2 and the bcl-2 transfectant clone 61 (27). Unless otherwise indicated, cells were cultured in RPMI 1640 containing 10% fetal calf serum in 2.5-mg/ml of Geneticin.

Assessment of Cytotoxicity. N10-Propargyl-5,8-dideazafolic acid (CB37717) (29) and an analogue which was not polyglutamated, ICI-M247496 (30), were a gift from ICI Pharmaceuticals, Macclesfield, United Kingdom. Cells were harvested from parental cultures after one subculture in Geneticin-free medium and were in logarithmic growth with <10% spontaneous apoptotic cells. They were seeded 2 × 105/ml (Day 0) and were incubated for 24 h prior to drug addition to ensure that bcl-2 transfectants and SV2 vector controls were both midway in their log phase of growth at the time of drug addition. After exposure to drugs for 36 h, cells were washed and resuspended at the original seeding density, using cell-free conditioned medium from stock cultures to avoid lag-phase kinetics. Morphological features of apoptosis were monitored by fluorescence microscopy, using acridine orange staining (5 μg/ml; Molecular Probes, Inc., Eugene, OR) as described previously (26). Cells were simultaneously examined for trypan blue exclusion (0.2%; Sigma). Experiments were carried out at least 3 times.

Flow Cytometric Analysis of Apoptosis. Apoptotic cells were quantitated using the flow cytometric assay exactly as described by Dive et al. (31). In brief, Hoechst 33342 (10 μM) (Molecular Probes) and propidium iodide (32 μM) were added to single cell suspensions 1 min before analysis of 106 cells, using a Coulter Epics V instrument with UV laser excitation (100 mW) at 357 and 337 nm. Forward angle light scatter, blue fluorescence (430 to 530 nm), and red fluorescence emissions were collected through a 630-nm long-pass filter. Cells (2 × 105/sample) were analyzed using a Coulter Epics V instrument with UV laser excitation (100 mW) at 357 nm and an excitation power of 337 nm. For simultaneous analysis of trypan blue exclusion (0.2%; Sigma). Experiments were carried out at least 3 times.

Cell Cycle Analysis. Flow cytometric analysis of propidium iodide-stained, fixed cell samples allowed determination of cell cycle profiles. For each sample, 1 × 106 cells were resuspended in 500 μl of 0.1% paraformaldehyde in PBS (pH 7.4) containing 0.1% Triton X-100 and stored at 4°C. Cell samples were stained with propidium iodide (100 μg/ml) for 1 to 2 min at room temperature prior to flow cytometric analysis using a Coulter Epics V machine (Coulter Electronics, United Kingdom). The cytometer was set to excite at 400 mW using the 488-nm line of the argon laser, and red fluorescence emissions were collected through a 630-nm long-pass filter. Cells (2 × 105/sample) were analyzed.

Quantitation of Thymidylate Synthase. TS activity was assayed in vitro by 3H release as previously described (17). This method utilizes the conversion of [1H]dUMP to H2O. For measurement of the amount of TS protein by Western blotting, 5 × 106 cells were washed once at 170 × g in PBS at 4°C, and the dry pellet was solubilized in 200 μl of reducing lysis buffer [50 mM Tris-HCl (pH 6.8); 2% SDS:0.1% bromophenol blue:10% glycerol]. Extracts were then subjected to SDS:12% polyacrylamide electrophoresis. Cell lysates from a thymidylate synthase-overexpressing cell line W1-L2:cl were used as a control. Western blotting was performed as described previously (32). After transfer, immunochromedical localization of protein involved incubation with a 1:1000 dilution of polyclonal antibody Ba30 (rabbit anti-human thymidylate synthase) in TMT for 2 h at room temperature. Filters were washed 5 times in TMT without Tween 20 and milk protein and then exposed to 1:16,000 goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma). TBS-washed filters were visualized by use of an ECL Western blotting detection system (Amersham International, Aylesbury, United Kingdom). Densitometric analysis was performed by using an LKB 2202 Ultrascan. All densitometric readings were normalized to the TS protein from the TS-overproducing W1-L2:cl human lymphoblastoid cell line.

Measurements of Intracellular dTTP and dUTP. Cells were seeded and allowed to enter the logarithmic phase of cell growth. 5-Fluorodeoxyuridine (1 μM) was added for the appropriate times prior to harvesting. Deoxyribonucleotides were assayed as previously described (33). Briefly, cells were extracted at the end of the drug exposure period using ice-cold 0.4 M perchloric acid and then treated with 0.5 M sodium periodate to remove cross-reacting ribonucleotides. The dUTP/TTP was purified by column chromatography, and the resultant preparation was assayed by radioimmunoassay (33).

Alkaline Elution. DNA strand breaks induced by exposure to 5-fluorodeoxyuridine were detected by alkaline elution (34). Since inhibition of TS causes deoxyribonucleotide imbalance and misincorporation of nucleotides during replication of DNA, DNA strand breaks were measured in newly replicated DNA (nascent DNA) as well as in mature DNA (see Ref. 22). For studies with newly replicated DNA, [14C]dThd (59.6 mCi/mmol) was added to 1 × 106 cells at 0.0825 μCi/ml during the last 4 h of drug exposure prior to alkaline elution. For studies of the integrity of mature DNA, cells were prelabeled for 24 h with [14C]dThd (0.0165 μCi/ml) and then incubated without radiolabel (a "cold chase") for various times prior to drug addition. In studies with both newly replicated and mature DNA, cells were lysed onto polycarbonate filters ( pore size, 2.0 μm; diameter, 25 mm; Nucleopore Sterilin, Middlesex, United Kingdom), and the lysis fraction was retained for counting. Alkaline elution was performed at pH 12.1 in the presence of proteinase K (0.5 mg/ml) using standard procedures (34).

RESULTS

bcl-2 Protein Expression and Cell Characteristics. Transfection of Burkitt's lymphoma Group I cells with the bcl-2 construct resulted in stable expression of the bcl-2 protein (Fig. 1). This clearly imparted a survival advantage to the transfected cells, since there was a dramatic reduction in the levels of spontaneous apoptosis as cell density increased in the untreated controls to reach the plateau phase of growth (Figs. 2 and 3). The expression of the bcl-2 protein in the transfects was approximately twice that of the Group III Burkitt lymphoma cells (Fig. 1), a subclone which constitutively expressed the gene (25) but which had become tetraploid. Analysis of the logarithmic growth rate of bcl-2-transfected cells and the vector controls showed these to be identical, with a doubling time of approximately 30 h (Fig. 2). Cell cycle analysis of cells in logarithmic growth showed that bcl-2 expression had not changed the distribution of the cells within the cell cycle. The mean proportions of cells in both the vector controls and the bcl-2 transfects in G1, S, and G2-M were as follows: 44%, 30%, and 26% (SEM = ±1.5%, n = 3).

Quantitation and Enzyme Activity of TS in bcl-2-transfected Cells. The results of Western blotting for TS are shown in Fig. 4. In the logarithmic phase of growth the amount of enzyme protein was identical in the vector controls and bcl-2 transfecants (as measured by densitometry). Estimation of the enzyme activity of TS also showed that this was not changed by the expression of bcl-2 protein. In two separate experiments, activity was 0.17 (SV2) and 0.18 (bcl-2), then 0.39 (SV2) and 0.33 (bcl-2), nmol of product formed/106 cells/h. The results of two separate experiments, activity was 0.17 (SV2) and 0.18 (bcl-2), then 0.39 (SV2) and 0.33 (bcl-2), nmol of product formed/106 cells/h.
Drug Treatment and Response. The numbers of total viable cells (which excluded trypan blue) and the percentage of apoptotic cells are shown in Figs. 2 and 3 for treatment with FdUrd and Fig. 5 for CB3717 and ICI M247496. Incubation with three concentrations of FdUrd significantly diminished the viability of the vector controls. This loss of viability was significantly reduced in the cells which contained the bcl-2 protein. Prior to drug treatment, cells in logarithmic growth phase showed basal, low levels of spontaneous apoptosis. These low levels were seen to continue during the first 12 h of drug treatment, after which the response of the cells diverged; i.e., SV2 vector controls began to undergo apoptosis at 24 h, whereas the bcl-2-transfected cells continued to exhibit only basal levels of apoptosis up to and including the time of drug removal (36 h). Cells which were washed and replaced in drug-free medium were observed to undergo further apoptosis. This was delayed and significantly lower in cells which expressed bcl-2. For example, Fig. 3 shows that 10 μM 5-fluorodeoxyuridine induced 90% apoptosis 24 h after removal of the drug in the vector control cells but only 18% in the bcl-2 transfects. Growth inhibition and delay of apoptosis followed the same pattern for the other two quinazoline inhibitors of TS (Fig. 5). The fall in the number of apoptotic cells from a peak of 68% at 84 h, after 2 μM CB3717, represents outgrowth of viable, nonapoptotic cells from the SV2 vector controls.

Apoptotic cell death was confirmed by flow cytometry as described by us previously using this cell type (31). Fig. 6 shows two dimensional frequency contour plots of blue fluorescence (Hoechst-bound DNA) versus FALS (cell size). A and B (untreated SV2 vector controls and untreated bcl-2 transfects, respectively) show the distribution of cells into three defined subsets, exactly as seen previously for these BL cells (31). Viable cells appear in Subset 1 with low blue fluorescence and high FALS. Apoptotic cells appear in Subsets 2 and 3 with increased blue fluorescence and decreased FALS. C and D show the effect of treatment with 1 μM FdUrd on the subpopulational distribution. Again, three clearly defined subsets were recognized. The fluorescence and scatter signals in the remaining viable cells were shifted upward and to the right (drug treatment increased both their Hoechst fluorescence and size). Apoptotic cells continued to exhibit a relative increase in blue fluorescence and a relative decrease in FALS compared to viable cells. In the representative experiment shown in Fig. 6, expression of bcl-2 decreased the amount of apoptotic cells seen 36 h after drug addition. Cell subpopulations were sorted for agarse gel electrophoresis of extracted DNA. However, after many attempts only faint DNA “ladders” were observed for cells sorted from Subsets 2 and 3 (data not shown). We consider that the background of DNA strand breaks imposed by thymidylate stress (see below) was superimposed on internucleosomal fragmentation patterns preventing good resolution of discrete oligonucleosomal bands of DNA.

When a study was made to establish the earliest time of appearance of apoptotic cells (by acridine orange staining), onset in vector control cells treated with 1 μM FdUrd occurred, during drug exposure, at 24 h (31%). Levels were at basal control levels after 12 h of drug...
covalent interaction between FdUrd and the thymidylate synthase enzyme gives rise to an
increase in the levels of dUTP pools. Densitometric analysis of two representative blots showed that incubation of the cells with FdUrd resulted in a rise in the number of nonviable cells as estimated by the exclusion of trypan blue. This is a process termed secondary necrosis (35). No apoptosis was observed in the bcl-2 transfects during the period of exposure to FdUrd. (This is discussed further, below, in the presentation of the analysis of changes in DNA integrity by alkaline elution).

Effects of TS Inhibitors on Burkitt’s Lymphoma Cell Metabolism. The increased survival of the cells which expressed bcl-2, after treatment with each of the TS inhibitors, may have been due to the modulation of a number of parameters which are critical for the expression of toxicity after TS inhibition, as discussed in the introduction. We consider that bcl-2 had not changed the transport of any of the agents across the cell membrane, since all of the agents inhibited growth in both the bcl-2-expressing and nonexpressing cells to the same degree. Moreover, ICI M247496 is a lipophilic TS inhibitor which enters the cell by diffusion (30); this was capable of an equal imposition of cytostasis in vector control cells and those which expressed bcl-2 (data not shown). Similarly, the expression of bcl-2 appeared not to have changed the cytotoxicity of the quinazoline-based inhibitor CB3717 because of changes in the ability of the cells to perform polyglutamation since neither ICI M247496 nor FdUrd undergo this modification. Western blotting to measure amounts of TS during incubation of cells with FdUrd (1 μM) indicated an increase in the expression of TS, presumably as the cells attempt to overcome thymidylate stress (Fig. 4). Densitometric analysis of two representative blots showed that there was an equally increasing amount of TS enzyme with time so that, by 36 h, the SV2 transfectants had increased the relative amount of total TS to 660% of the control zero time value and, in the bcl-2 transfectants, this had increased to 640% of control value. This phenomenon has been reported previously (36–38). Interestingly, there was no difference in the rate or extent of TS upregulation in vector control or bcl-2-expressing cells even up to the point where they initiate apoptosis. Inspection of the Western blot also showed that incubation of the cells with FdUrd resulted in the appearance of equivalent amounts of a higher molecular weight form of the enzyme (Fig. 4). This may correspond to the formation of the ternary complex between the drug and a subunit of the enzyme to give a protein with a molecular weight of approximately 38,500, as has been observed recently by Johnston et al. (16). Significantly, the rate of formation of this form of TS was the same in both the SV2 vector controls and the bcl-2 transfectants, indicating that formation of drug-receptor/enzyme complex was not influenced by expression of bcl-2. Similarly, there were no differences between changes in nucleotide pools in the two cell types during FdUrd treatment. In untreated cells, the mean TTP concentration was 24.8 pmol/10^6 cells in the bcl-2 transfects and 19.5 pmol/10^6 cells in the SV2 vector controls (n = 2). The concentration of TTP fell equally in both cell lines after treatment with 1 μM FdUrd, so that this was 21% and 26% of control level after 6 h, for the bcl-2 transfectants and SV2 transfectants, respectively, falling to 4% and 5% at 24 h, and to below detectable levels (detection limit, 1 pmol/10^6 cells) in both by 36 h. Significant changes in dUTP pools were not observed in either cell line. Equivalence in the depression of TTP pools was reflected in exactly parallel increases in the utilization of radiolabeled thymidine from the culture medium when [1^4C]TTP was incorporated into DNA during the last 4 h of drug exposure, prior to alkaline elution of nascent DNA (see “Materials and Methods”). Twenty-four h after 1 μM FdUrd, SV2 transfectants had accumulated 215,000 dpm, and the bcl-2 transfectants, 217,000 dpm, in a representative experiment.

Analysis of DNA Integrity, by Alkaline Elution. The imposition of thymidylate stress by FdUrd induced strand breaks in nascent DNA of both control and bcl-2-expressing cells. These strand breaks increased with time of exposure to the drug (Fig. 7), were quantitatively related to drug concentration (data not shown), and accumulated at an equivalent rate prior to apoptosis. At 24 h and 36 h, however, it appeared that the bcl-2 transfectants had more strand breaks than did the vector controls. Analysis of the integrity of mature DNA (i.e., that made prior to drug exposure, see “Materials and Methods”) showed no effect of FdUrd at concentrations of up to 10 μM for 24 h if the lysis fraction was not included, but thereafter breaks were observed (data not shown). The elution profiles obtained for the vector control cells show that, with the onset of apoptosis, there was an increase in the fraction of DNA which eluted rapidly through the filters during lysis which we and others (39) presume to be fragments generated by the activity of the apoptosis-associated endonuclease. This constituted 60% of the total counts at 36 h in the SV2 vector control cells treated with 1 μM FdUrd and compares well with the percentage of apoptotic cells observed at this time (Fig. 3). This loss of DNA in the lysis of vector control cells at times greater than 24 h may explain why apparently more strand breaks are formed in the nascent DNA from bcl-2 transfectants.

![Fig. 4. Western blot analysis of cell lysates from SV2 and bcl-2-transfected MUTU BL Group I cells to show the TS content in the transfecants and changes after continuous treatment with 1 μM FdUrd (see “Materials and Methods”). The TS protein has a molecular weight of approximately 36,000. The ternary complex which is formed by the covalent interaction between FdUrd and the thymidylate synthase enzyme gives rise to an apparently higher molecular weight form of the enzyme as determined by SDS polyacrylamide gel electrophoresis. Cell lysates from a TS-overexpressing cell line W1-L2:c1 were included on each immunoblot as an internal standard.](image_url)
M-2-INDUCED DRUG RESISTANCE

Fig. 6. Flow cytometric analysis of spontaneous (A and B) and FdUrd-induced (C and D) apoptosis in MUTU BL Group I cells transfected with the SV2 vector construct (A and C) and bcl-2 (B and D) using the method described by Dive et al. (31). Cells were treated with 1 μM FdUrd of vehicle control for 24 h, and 20,000 cells were analyzed 36 h after drug addition. Data are displayed as two-dimensional frequency contour plots, where viable cells appear in Subset 1, and those undergoing apoptosis display increased blue fluorescence (arbitrary units, Hoechst 33342-bound DNA) and decreased forward light scatter (arbitrary units, a measure of cell size) and appear in Subsets 2 and 3. Cells which allow uptake of propidium iodide have been excluded from the analysis. Results shown are from a representative experiment taken from 3 independently repeated studies.

DISCUSSION

Considerable excitement has been engendered by recent reports which claim that expression of the protooncogene bcl-2 inhibits drug-induced apoptotic cell death and provides resistance by an entirely novel mechanism (11–14). In the absence of data which demonstrate that the classical and well-defined determinants of drug resistance do not operate, these claims for a novel mechanism of drug resistance must be treated with some caution.

Mechanisms of drug resistance to antimetabolites, such as to the inhibitors of the enzyme thymidylate synthase, have been well defined (reviewed in Refs. 18 and 40). Alterations in the qualitative and/or quantitative aspects of the drug target, through mutation, deletion, or amplification, or of factors which limit the quantitative or temporal aspects of the drug-target interaction, for example, by limitation of intracellular accumulation of the drug, are the major determinants of drug activity. The ability of the cell to repair damage, such as to the genome, also influences the outcome of treatment. The molecular determinants of what may be termed the “inherent” resistance of some cells have not been defined, although modulation of so-called “downstream” events of the immediate effects of drug action is now being considered as the arbiter of a cellular response (6, 7).

This study asked the question whether bcl-2 expression modulated classical mechanisms of resistance to TS inhibition. It does not. Neither the amount (Fig. 4) nor the enzyme activity (“Results”) of the TS target was altered. The drugs effectively inhibited cell growth to an equal extent (Fig. 2; “Results”), reflected in equivalent falls in the TTP pool (“Results”) and, in the case of fluorodeoxyuridine, imposed similar amounts of DNA damage (Fig. 7). However, bcl-2 expression afforded protection against the cytotoxicity of all the TS inhibitors studied. Our data, when combined with those of others (8–14), strongly suggest that the expression of bcl-2 provides a resistance to cytotoxins which is independent of the locus of action.

Fig. 7. Alkaline elution of newly synthesized (“nascent”) DNA from MUTU BL Group I cells transfected with the SV2 vector construct (a) or the bcl-2 gene (b) and treated for various times (0 to 36 h) with 1 μM FdUrd (see “Materials and Methods”). The graphs show the fraction of new [14C]dThd-labeled DNA retained on the filter at the time intervals shown on the x-axis.
of the toxin and may therefore be pleiotropic. Because the function of bcl-2 has not been determined, it is not yet possible to define how its expression protects against cell death initiated by a thymidylate stress or other types of toxicity. The protection observed here was related to drug concentration (Figs. 2 and 3) and, when modest amounts of cell kill were induced (Figs. 2 and 5), survivors repopulated the cultures, giving a growth delay which was shorter than that observed after treatment of the vector controls. Unfortunately, we were unable to clone the vector control cells and therefore were unable to establish whether bcl-2 expression enhanced clonogenic potential after treatment with TS inhibitors, a critical question. The data presented here show a distinctive delay in the onset of apoptosis, and whether this provides time for repair and later regrowth remains to be established. Miyashita and Reed (13) showed that, after treatment of another human B-cell leukemia which expressed bcl-2, there was a clonogenic advantage after methotrexate treatment. In experiments in progress, we have found that refeeding cells with fresh serum 6 days after treatment with 1 μM FdUrd (see Fig. 2) resulted in an increase in cell numbers in the bcl-2 transfecteds, reflecting a reducing rate of apoptosis and the emergence of proliferating cells. We have now transfected the readily clonable and transplantable murine leukemia cell line L1210; this will allow investigation of the events occurring as cells presumably repair damage and recover their proliferative and clonogenic capacity. It is possible that the survival of DNA-damaged cells, and the delay in onset of apoptosis promoted by expression of bcl-2, may lead to increased mutation of the genome and the later emergence of cells with greater oncogenic potential and/or drug resistance. An increase in homologous recombination activity has been shown to develop during FdUrd-induced thymidylate stress in mouse FM3a cells (41).

The activity of other cellular genes, such as c-myc and p53, is also implicated in determining the ability of some cells to engage apoptosis (reviewed in Ref. 42). Understanding how the program of apoptosis is initiated in response to toxins like the TS inhibitors and how expression of a gene like bcl-2 can attenuate this should provide insights into the molecular basis of pleiotropic drug resistance.

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REFERENCES


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